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Award Number: DAMD17-00-1-0580

TITLE: Control of Alcoholism-Related Folate Deficiency by Regulation of Urinary Folate Excretion

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REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20020124 382

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (01 Oct 00 - 30 Sep 01)

4. TITLE AND SUBTITLE
Control of Alcoholism-Related Folate Deficiency by Regulation of Urinary Folate Excretion

5. FUNDING NUMBERS
DAMD17-00-1-0580

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Health-related consequences of heavy drinking include nutritional deficiencies. Folate deficiency occurs from multiple causes including increased urinary folate excretion. Ethanol decreases the renal reabsorption of folate leading to increased excretion. The main aim of this research is to determine the mechanism by which ethanol decreases folate transport by the kidney. An initial objective is to determine the effects of ethanol on expression of the two renal transport proteins, the folate receptor (FR) and the reduced folate carrier (RFC). 5 day treatment with ethanol in vitro increased the content of both transporters in cultured human proximal tubule cells. 14 day treatment of rats in vivo with ethanol-containing diets increased the content of both transporters in isolated rat kidney membranes. This increase in transport protein content with repeated ethanol treatment may represent a way to counteract the acute ethanol-induced decrease in folate uptake in order to restore folate homeostasis. The results indicate that the long term increase in folate excretion does not result from a down-regulation of the folate transport proteins. Subsequent studies will use transport kinetic studies and pathway inactivation studies to determine which transporter is affected by ethanol.

14. SUBJECT TERMS
alcoholism, chronic ethanol abuse, urinary folate excretion, folate deficiency, folate transport proteins, cultured kidney cells

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Recent surveys have shown that military personnel are more likely to drink heavily (≥ five drinks at least once per week) than civilian controls (1). Although overall alcohol use has declined, nearly 20% of military personnel engage in heavy drinking and alcohol-related consequences continue to be a problem. Health-related consequences include alcohol-induced nutritional deficiencies such as deficiency of the vitamin folic acid (2), which can lead to megaloblastic anemia, to neural tube birth defects or possibly to atherosclerotic disease. In animals, ethanol treatment markedly increases urinary folate excretion by decreasing renal folate reabsorption(3,4), thereby contributing substantially to the folate deficiency. These effects are observed at blood levels in the range of 150-300 mg/dl (5,6), which are often reached in heavy drinking humans. Acute ethanol administration decreases the apical (AP) transport of the physiologic folate, 5-methyl-tetrahydrofolate (5M), by normal human proximal tubule (HPT) cells in culture. The two pathways known to be involved in the AP transport of 5M by cultured proximal tubule cells are the folate receptor (FR) and the reduced folate carrier (RFC) (7). The primary objective of this research is to determine the mechanism by which ethanol alters 5M transport by the proximal tubule cell since urinary folate excretion is primarily regulated by the reabsorption of 5M from the tubular lumen. Our hypothesis is that ethanol decreases the AP-mediated transport of 5M (hence its reabsorption) by decreasing the activity in either the FR or RFC pathway. After either acute or chronic ethanol treatment, 5M transport will be studied in HPT and rat PT cells, cultured on membrane filter inserts to maintain distinct AP and basolateral (BL) compartments (8). The major aims will be to determine which pathway is affected by ethanol using a combination of kinetic studies, pathway inactivation studies and immunoblotting studies of the content of each of the proteins. Hence, these studies will provide mechanistic information that is needed to design interventions to reduce the morbidity from the folate deficiency that results from chronic heavy drinking.

BODY

Technical Objective 1. **To characterize the effects of acute and chronic ethanol on bidirectional renal folate intracellular transport.** In the Statement of Work, this objective was to be accomplished in Years 1 and 2. Our progress to this objective was initially slow, but has recently become satisfactory. To accomplish this goal, my first action was to recruit a postdoctoral fellow. Through a combination of advertisements, registration with placement services and attendance at scientific meetings in the area of research, I interviewed a number of candidates early in the year. However, none of them were able and willing to work in my laboratory right away. Finally, in August, I was able to attract Dr. Sivakumar J-T-Gowder from UT Southwestern in Dallas, TX to start working on this project. Dr. J-T-Gowder's research experience includes studies on the expression of genes involved in renal hypertrophy (in Texas) and on the nephrotoxic potential of food additives (previously in India). Thus he is well-versed in studies on the kidney as well as nutritional studies. His experience will be valuable for this project.

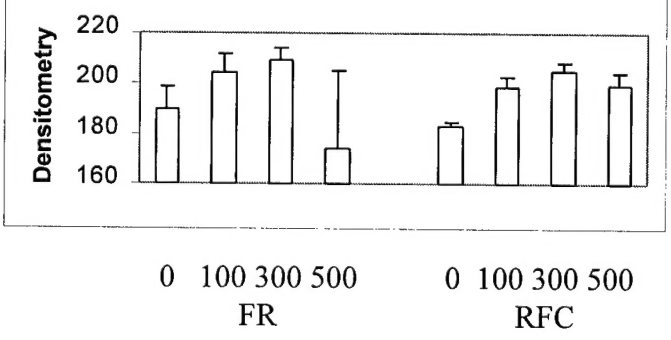
In the two months of his work, I have trained Sivakumar in cell culture techniques and in techniques involved in kinetic studies of folate transport. He has developed the ability to culture rat PT cells, which is a necessary focus of this objective. We are beginning to train him in the HPLC analysis of folate metabolites, which is also a major focus of this objective. Although he has not generated reportable data on this objective, he has rapidly learned the necessary techniques and

progress in the second year will be fast.

Technical Objective 2. **To characterize the interaction of ethanol with the renal folate receptor (FR), as a mechanism by which urinary folate excretion could be affected.**
Technical Objective 3. **To characterize the effects of ethanol on the renal reduced folate carrier (RFC) activity, as a mechanism by which urinary folate excretion could be affected.** These objectives are discussed together since they are being tested simultaneously, with the Statement of Work stating that the acute effects of ethanol on renal FR and RFC expression in cells and in vivo will be tested in Year 1. We have made significant progress on these studies this past year.

Effect of repeated ethanol treatment on FR and RFC expression in HPT cells. Because our preliminary studies had shown that acute treatment of HPT cells with high concentrations of ethanol decreased intracellular uptake of folate, we conducted follow-up studies to measure the effects of ethanol on the content of the two folate transporters in the AP membranes of HPT cells. Confluent cultures of HPT cells were subcultured using trypsin-EDTA and seeded into tissue culture flasks (75 cm^2). These cultures were incubated for about 5 days to allow cells to grow to near confluency. At that point, the growth media were changed in respective flasks to the same media containing additional ethanol in concentrations of 0, 100, 300 and 500 mg/dL (0, 22, 65 and 109 mM). Cells were exposed to ethanol for five days, by replacing the media daily to ensure continued exposure to the expected ethanol levels. Flasks were also sealed in plastic boxes while in the incubator in order to maintain consistent ethanol exposures. After 5 days, the cells were removed from the flasks by scraping into "Sucrose" buffer (0.25 M sucrose, 10 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.0, containing 1 mM aminoethylbenzenesulfonylfluoride (AEBSF), 100 μM leupeptin and 10 μM bestatin as protease inhibitors). Cells were homogenized in glass homogenizers and AP membrane fractions were obtained by differential centrifugation (9). The resulting pellets were resuspended in sucrose buffer containing fresh protease inhibitors and stored frozen for analysis. The membrane fractions were thawed, solubilized in 2% SDS and subjected to SDS-PAGE prior to transfer to PDVF membranes for immunoblot analysis (10). After blocking to minimize nonspecific binding, the membranes were treated with primary antibodies to either the FR (a rabbit polyclonal anti-human placental FR preparation) or the RFC (a rabbit polyclonal anti-human recombinant RFC preparation). Membranes were then treated with anti-rabbit secondary antibodies and the complexes were detected by enhanced chemiluminesence, followed by exposure to film. The bands were quantitated by computer-assisted densitometry.

Figure 1 - Effect of ethanol on FR and RFC content in HPT cells

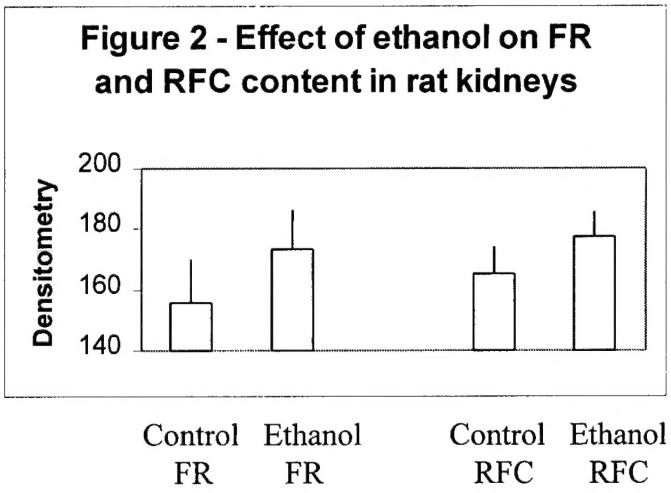


Reproductions of the original blots are shown in the Appendix. The corresponding densitometry scans are summarized in Figure 1 to the left. For each concentration, the n = 2. We are processing other samples, so will have an n of at least 5. Although the result are sketchy, it appears that the repeated ethanol treatment increases the content of the RFC in the AP membranes of HPT cells, with effects at 100 mg/dL and higher. A similar

trend is seen for the FR content, with an increase at concentrations $\geq$ 100 mg/dL (the high variability at 500 mg/dL makes a firm conclusion difficult at this time). This appears to represent an up-regulation of both folate transport pathways. Such an up-regulation might be expected as an adaptive response to the previously-shown decrease in folate uptake by acute ethanol treatment. That is, the cells increase the content of the folate transporters to counteract the initial decrease in activity of the folate uptake pathways due to ethanol exposure. This adaptation returns the amount of folate uptake towards normal. These studies also suggest that the inhibitory effect of ethanol on transport is not likely due to a decrease in expression of a folate transport protein.

Effect of repeated ethanol treatment on FR and RFC expression in rat kidneys in vivo. Acute ethanol treatment of rats is known to increase urinary folate excretion about 6-fold within 4-8 hours. (3) Chronic ethanol treatment of rats also increases urinary folate excretion within two weeks by about two-fold levels (5). In order to examine whether ethanol affects urinary folate excretion by altering the expression of the RFC or FR in the AP membranes of the rat kidney in vivo, we conducted the following study. Sixteen male Sprague-Dawley rats (about 250-300 g) were acclimated to control liquid diets (Lieber-DeCarli formula (5)) for a period of 4 days, then were divided into two groups of 8 rats, fed either an ethanol diet (36% of calories, 5% w/v) or a control diet (isoenergic carbohydrate to replace ethanol). Each control animal was fed its diet in a pair-fed manner to a corresponding ethanol rat (restricted to the previous day's consumption of its pair) in order to maintain consistent nutrient intake between groups. Food consumption and rat weights were obtained daily. Diets were replenished in the late afternoon of each day to ensure that the rats received fresh diets and proper nutrient intake. On Day 13, rats were removed from their home cages and placed in metabolic chambers for 24 hours. Rats had continued access to their diets in the chambers. Urine was collected at 8 h intervals into tubes that contained 2-mercaptoethanol and were wrapped in foil to minimize oxidation of folates. The 3 samples were combined for a 24 h sample, then stored frozen for folate analysis by microbiological assay. The rats were returned to their home cages for the 14th day. On the morning of the 15th day, rats were sacrificed by decapitation and the trunk blood was collected into tubes containing heparin as anticoagulant. The kidneys were then rapidly removed and stored at -20^{o} C. An aliquot of the blood was removed, deproteinized and centrifuged, with the supernatant stored frozen for analysis of ethanol level by gas chromatography; the remaining blood was centrifuged to obtain the plasma, which was treated with 2-mercaptoethanol and frozen for analysis of total folate level by microbiological assay. The cortex was removed from the stored, partially thawed kidneys by careful excision, then was homogenized using a tissuemizer in sucrose buffer (as above, except containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as the protease inhibitor). The AP membranes were recovered by differential centrifugation (9) and were solubilized in SDS and prepared by immunoblotting to measure FR and RFC content, as described above for the HPT cell membranes.

Reproductions of the original blots are shown in the Appendix. The corresponding densitometry scans are summarized in Figure 2 below. The results indicate that the 2 week ethanol treatment increased the content of both the FR and the RFC in the AP membranes of rat kidneys. Similar to the HPT cells, these results suggest that the rat kidney responds to increase the amount of the folate transporters as a way to counteract the acute effect of ethanol (which is to decrease the reabsorption of folate from the lumen leading to increased urinary folate excretion. The fact that acute ethanol increases excretion about 6-fold, while chronic treatment increases it only about 2-fold indicates that one would expect some compensation to have occurred in the chronic animals. Our results suggest that an up-regulation of the transporters is the mechanism for this compensation.



KEY RESEARCH ACCOMPLISHMENTS

- Recruitment and training of a postdoctoral fellow, who has begun to conduct kinetic and pathway inactivation studies to determine whether the FR or RFC pathway is the one affected by ethanol.

- Exposure of HPT cells to ethanol for 5 days may suggest an up-regulation of both folate transporters, the FR and the RFC. This effect may be concentration related, with effects on the RFC appearing at levels $\geq$ 100 mg/dL, while the effects on the FR are less certain. The increase in folate transporter content in the cell membranes may be a compensatory reaction to overcome the ethanol-induced decrease in folate transport into the cells.

- Treatment of rats with ethanol in the diet for 14 days increases the FR and RFC levels in the AP membranes of the rat kidneys. This increase in transporter content should counteract the decrease in folate reabsorption (increase in urinary excretion) that occurs due to ethanol and may represent an adaptive response.

REPORTABLE OUTCOMES

Abstracts

1. Romanoff RL, Suvarna S, McMartin KE. Regulation of folate transporters in human kidney tissues by nutritional alteration. Graduate Research Day, LSU Health Sciences Center, Shreveport, May 18, 2001.

CONCLUSIONS

We have made reasonable progress towards the technical objectives outlined in the Statement of Work. We expected to measure the effects of ethanol on renal FR and RFC expression in cells and in vivo and we have almost finished these studies. We expected to characterize the folate transport kinetics and to measure the folate metabolites to assess how ethanol affects these processes

in the kidney. We have not yet accomplished this task due to an initial lack of trained personnel. However near the end of year 1, a postdoctoral fellow was hired and is currently being trained in the necessary techniques. Since these studies were designed to be completed in year 2, we are still on schedule, assuming a rapid response by the fellow.

Repeated ethanol treatment of kidney cells in culture and chronic ethanol treatment of rats in vivo appears to increase the content of the two folate transport proteins, the FR and the RFC, in the AP membranes of the kidney. Initially this increase in folate transporter content would seem to be counterintuitive since such ethanol treatments are known to decrease the activity of AP-mediated folate transport. The increase in protein content could be explained as an adaptive response by the cell or the organism to counteract the decrease in folate uptake. In such a way, the cells react to the initial decrease in folate uptake by up-regulating the overall number of transporters. The increased number of transporters would then act to restore the level of folate uptake towards normal. This hypothesis is backed by previous data on folate excretion - after one ethanol treatment, urinary folate excretion is increased to a much larger extent than after two weeks of ethanol exposure, suggesting an adaptive response.

"So what". Although these studies have produced interesting results, we have not yet determined the mechanism by which ethanol initially produces the decrease in renal cell uptake of folate. Hence, our studies in the coming year will be focussed on determining which pathway, either the FR or the RFC is decreased by ethanol. As originally outlined in the proposal, we will use specific substrates to measure each pathway and also specifically inactivate each pathway with covalent inhibitors prior to the ethanol treatments in order to assess which pathway is affected by ethanol.

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APPENDICES

1. Copy of Graduate Research Day abstract
2. Immunoblots of the effects of 5-day ethanol treatment of HPT cells on FR (top) and RFC (bottom) contents.
3. Immunoblots of the effects of chronic ethanol diet treatment of rats on FR (top) and RFC (bottom) contents.

Graduate Research Day 2001

May 18, 2001

Shreveport

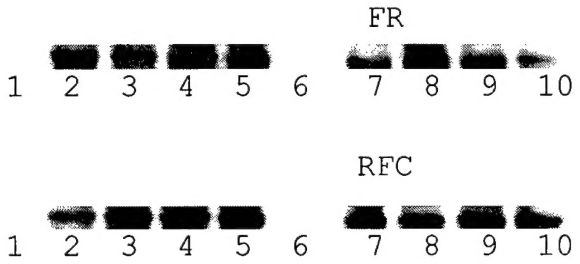
Regulation of Folate Transporters in Human Kidney Tissues by Nutritional Alteration

Romanoff, R.L., Suvarna, N., & McMartin, K.E.
Department of Pharmacology, LSU Health Sciences Center

Folate is a water-soluble B vitamin that is essential for cell metabolism and normal growth. The major physiologic consequences of folate deficiency include megaloblastic anemia, neural tube defects in the folate-deficient fetus, and hyperhomocysteinemia, the latter of which is correlated with an increased susceptibility to cardiovascular disease. The causes of folate deficiency include inadequate dietary intake (50-400 μg), intestinal malabsorption, altered hepatic metabolism, and increased urinary and fecal excretion. The mechanisms for maintaining folate homeostasis in normal tissues is not well understood despite the important physiological roles for folate. Two folate transport systems are considered to be responsible for regulating folate homeostasis. These transporters include the membrane-associated folate receptor (FR) and a glycosylated integral membrane protein, the reduced folate carrier (RFC). The hypothesis is that urinary folate excretion is increased following a decreased reabsorption of folate in the renal proximal tubule (PT) via the folate receptor (FR)- or reduced folate carrier (RFC)-mediated transport pathways. Levels of membrane-associated receptors and transport proteins may be upregulated or downregulated depending upon the availability of their effector molecules. The levels of the folate transporters could therefore be affected by the availability of folates to the cell.

Previous studies in cultured human carcinoma cells have demonstrated that low extracellular folate concentrations (2-10 nM) result in an upregulation of the FR protein as determined by Western blotting. Our lab performed this experiment in normal tissues using cultured human proximal tubule (HPT) cell isolates. The cells were passaged in either high folate (2 μM) or low folate (20 nM) media, after which the cells were examined for FR and RFC protein content via Western blotting. Our results demonstrate that the expression of the FR protein is increased at least 3-fold over control and that the RFC expression is increased about 2-fold over control. The molecular upregulation of the FR and RFC transporters corresponds to the increased transport of folate that was observed in prior studies. Our results indicate that HPT cells cultured in low folate media undergo an upregulation of both the FR and RFC transporter proteins to maintain folate homeostasis.

Effects of 5-day Ethanol Treatment
of HPT Cells on FR and RFC Contents



Appendix 2. Lane 1: standard molecular weight markers; Lane 2: 0101 isolate, 0 mg/dl; Lane 3: 0101 isolate, 100 mg/dl; Lane 4: 0101 isolate, 300 mg/dl; Lane 5: 0101 isolate, 500 mg/dl; Lane 6: Blank; Lane 7: 0102 isolate, 0 mg/dl; Lane 8: 0102 isolate, 100 mg/dl; Lane 9: 0102 isolate, 300 mg/dl; Lane 10: 0102 isolate, 500 mg/dl.

Effects of Chronic Ethanol Diet
Treatment of Rats on FR Content

Appendix 3. S = Standard molecular weight markers, B = Blank, C = Control rats, E = Ethanol-treated rats.

Effects of Chronic Ethanol Diet
Treatment of Rats on RFC Content

Appendix 3. S = Standard molecular weight markers, B = Blank, C = Control rats, E = Ethanol-treated rats.